AD-A246 026

CUMENTATION PAGE

Form Approved
OMB No. 0704-0188

2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
1990	Reprint

4. TITLE AND SUBTITLE

(see title on reprint)

5. FUNDING NUMBERS

PE: NWED QAXM

WU: 00020

6. AUTHOR(S)

Kiang et al.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Armed Forces Radiobiology Research Institute
Bethesda, MD 20889-5145

8. PERFORMING ORGANIZATION REPORT NUMBER

SR91-48

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Defense Nuclear Agency
6801 Telegraph Road
Alexandria, VA 22310-3398

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release; distribution unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

14. SUBJECT TERMS	15. NUMBER OF PAGES 11
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED		

CLASSIFIED BY:

DECLASSIFY ON:

Heat induces intracellular acidification in human A-431 cells: role of Na^+-H^+ exchange and metabolism

JULIANN G. KIANG, LESLIE C. McKINNEY, AND ELAINE K. GALLIN
Department of Physiology, Armed Forces Radiobiology Research Institute, Bethesda, Maryland 20814-5145

KIANG, JULIANN G., LESLIE C. McKINNEY, AND ELAINE K. GALLIN. *Heat induces intracellular acidification in human A-431 cells: role of Na^+-H^+ exchange and metabolism.* Am. J. Physiol. 259 (Cell Physiol. 28): C727–C737, 1990.—The resting intracellular pH (pH_i) of A-431 cells at 37°C in Na^+ Hanks' solution is 7.23 ± 0.02. In the presence of amiloride (100 μM) pH_i decreases to 7.08 ± 0.03. Hyperthermia induces a temperature- and time-dependent intracellular acidification of 0.2 pH units in either bicarbonate-free or bicarbonate-buffered solutions. After heat treatment (45°C, 10 min) pH_i returns to normal 1 h after incubation at 37°C. The activity of the Na^+-H^+ exchanger was examined in heated and unheated cells in the absence of bicarbonate. Unheated cells recover from an acid load in a $[Na^+]_o$-dependent and amiloride-sensitive manner. The apparent Michaelis constant for extracellular Na^+ is 38 ± 9 mM, and the apparent mean affinity constant for amiloride is 11 ± 3 μM. In heated cells the apparent affinity of the Na^+-H^+ exchanger for extracellular Na^+ is not changed, but the maximal recovery rate is ~40% slower than that of unheated cells. The rate of recovery from acid loading returns to normal 2 h after heat treatment. $[Na^+]_i$ and intrinsic buffering power in heated cells are the same as those in unheated cells. Decreases in both intracellular ATP and lactic acid are observed in heated cells. 2-Deoxy-D-glucose and sodium azide induce an intracellular acidification but prevent most of the acidification induced by heat. Heat treatment causes no further acidification in cells that are acidified by both amiloride and 2-deoxy-D-glucose together. These data are the first to suggest that thermally induced intracellular acidification is due to both an inhibition of Na^+-H^+ exchange and an inhibition of metabolic pathways.

intracellular pH; hyperthermia; epithelial cells; metabolic inhibitors; thermal injury; 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein; adenosine 5′-triphosphate; intracellular sodium concentration; buffering power; proton efflux

HEAT STRESS induces several different cellular responses, including inhibition of cell growth (21), activation of heat-stress gene expression (43), and synthesis of heat-shock proteins (43). The mechanism(s) by which heat-shock responses are induced has not been elucidated, although a number of intracellular parameters change following heat treatment, including intracellular pH (pH_i). pH_i is known to modulate DNA replication and cell proliferation in some cell systems (19). In mutant fibroblasts that lack a functional Na^+-H^+ exchanger, growth factor-induced alkalinization in bicarbonate-free buffer is blocked and cell growth is inhibited, although cell growth is normal in these cells in bicarbonate-containing medium (35). Furthermore, in 3T3 and Vero cells, the expression of a yeast proton pump, which results in an increase in pH_i of 0.2 units, is sufficient to induce tumorgenicity and growth in suspension culture (34). Additionally, it has been reported that pH_i has important effects on second messenger levels. In avian heart fibroblasts (8) and rat hepatocytes (47), an intracellular acidification induces a decrease in cytosolic Ca^{2+} and intracellular adenosine 3′,5′-cyclic monophosphate (cAMP) levels, and intracellular alkalinization causes an increase in cytosolic Ca^{2+} and intracellular cAMP levels even in the presence of $NaHCO_3$ (5 mM). These findings demonstrate that pH_i can affect cell growth, intracellular cAMP levels, and Ca^{2+}. Therefore, changes in pH_i following thermal injury may also affect these parameters.

The relationship between temperature and pH_i has not been examined in human cells, but in cells from other species there is a variable relationship between temperature and pH_i (see review in Ref. 36). For example, in giant barnacle muscle fibers (23) and mouse soleus muscle (1), pH_i falls linearly as the temperature increases from 5 to 40°C. Similarly, pH_i decreases when mouse mastocytoma cells are exposed to 43°C for 1 h (48), when salivary gland cells of *Drosophila* are heat treated at 35°C for 20 min (9), and when yeast cells are heat treated at 40°C for 45 min (46). In contrast, the pH_i of rat ventricular cells is unaffected by temperatures from 25 to 35°C (11), and no pH_i changes are found in murine NG108–15 neuroblastoma cells exposed to 43.5°C for 1 h (7). In Chinese hamster ovary cells, no pH_i changes are found following heat treatment at 43.5°C for 55 min. However, an intracellular acidification is found in these cells in the absence of external Na^+. Interestingly, an intracellular alkalinization is noted when these cells are acid-adapted before heat treatment (6).

In those studies the mechanism(s) underlying the heat-induced pH_i changes was not delineated. It is possible that thermal injury may directly affect the ability of cells to regulate pH_i. That is, heat treatment may modify the different ion transporters that are involved in pH_i homeostasis, including Na^+-H^+ exchange and Na^+-dependent and Na^+-independent Cl^--HCO_3^- exchange (18, 19). Alternatively, heat-induced pH_i changes may result from heat-induced metabolic inhibition. Several metabolic inhibitors have been shown to produce an intracellular acidification (3, 10, 32), although the effect of thermal injury on metabolism has not been characterized.

This study examines the effects of thermal injury on

pH_i of human epidermoid carcinoma A-431 cells and investigates the mechanisms underlying the pH_i changes that occur following heat treatment. We studied an epidermal cell line because skin cells are especially vulnerable to heat shock following burn injuries. A-431 cells were selected for these studies for several reasons. They have been used as an epidermal cell model in studies on the effects of epidermal growth factor (12, 41, 42). In addition, a Na^+-dependent amiloride-sensitive Na^+-H^+ exchanger has been identified in these cells (28, 37, 38), and its role in regulating pH_i has been partially characterized (37, 38). Finally, the effect of heat treatment on the metabolism of a tumor cell line is of interest to those who use hyperthermia as an adjuvant treatment in cancer therapy (21, 24), since the inhibitory effect of hyperthermia on the growth of tumor cells may be related to changes in pH_i.

METHODS

Cell culture. Human epidermoid carcinoma A-431 (American Type Culture Collection, Rockville, MD) were grown in 75-cm^2 tissue culture flasks (Costar, Cambridge, MA) in a 5% CO_2 incubator at 37°C. The tissue culture medium was Dulbecco's modified Eagle's medium supplemented with 0.03% glutamine, 4.5 g/l glucose, 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 10% fetal bovine serum, 50 μg/ml penicillin, and 50 U/ml streptomycin (GIBCO Laboratories, Grand Island, NY). Cells were fed every 3–4 days. Cells from passages 28–35 were used for experiments.

Heat treatment was performed by adding Hanks' solution at 37, 42, 45, or 50°C to the cells and placing the cells in the same temperature water bath for 10 s or 5, 10, 20, or 30 min. Cell viability was determined in three different ways: trypan blue exclusion, ethidium bromide-acridine orange staining, and lactate dehydrogenase release (13). The plating efficiency of heat-treated cells was examined by trypsinizing cells after heat treatment, replating, and 24 h later counting the percentage of adherent cells.

pH_i measurements. Confluent epithelial cells were trypsinized with 0.025% trypsin containing 0.2 mg/ml EDTA (Biofluid, Rockville, MD) for 10 min at 37°C. The cells were centrifuged and resuspended in Hanks' solution containing 5 mM glucose and 0.2% bovine serum albumin at 37°C for 1 h to allow them to recover from trypsinization (30). After this, the cells were centrifuged again, resuspended to 2×10^6 cells/ml in Hanks' solution supplemented with 5 mM glucose, 0.2% albumin, and 2 μM 2′,7′-bis(carboxyethyl)carboxyfluorescein acetoxymethyl ester (BCECF/AM), and incubated for 30 min at 37°C. At the end of the incubation period cells were washed twice with Na^+ Hanks' solution. Aliquots of cells were centrifuged, resuspended to 10^6 cells/ml in Na^+ Hanks' solution without glucose and albumin, and transferred to a cuvette maintained at 37°C. The BCECF fluorescence was measured with an SLM 8000C spectrofluorometer as the ratio of emission at 530 nm for dual excitation at 497 and 437 nm (slit width 4 nm). BCECF leaks out of A-431 cells at a rate of 0.77 ± 0.26%/min (n = 3) and 2.18 ± 0.11%/min (n = 3) at 37 and 45°C, respectively. Cells exposed to 45°C for 10 min and then returned to 37°C have a leak rate of 0.54 ± 0.05%/min (n = 3) that is not significantly different from the rate observed in unheated cells. The extracellular dye carried over from washes is 6 ± 1% (n = 3). The fluorescence signal was calibrated using a method modified from Thomas et al. (44) and summarized as follows. Cells were suspended in K^+ Hanks' solution {containing (in mM) 145 KCl, 5 NaCl, 1.2 $MgCl_2$, 1.6 $CaCl_2$, and 10 HEPES, pH 7.35 at 24°C; 145 mM K^+ is near the normal $[K^+]_i$ for these cells: $[K^+]$ = 137 ± 13 mM}. Nigericin (3 μM) and valinomycin (3 μM) were added to the cell suspension for 5 min. The solution was then titrated with KOH or HCl and the fluorescence signal recorded. Extracellular pH (pH_o) was recorded using a Corning model 125 pH meter. The calibration curves were fitted by a sigmoid line, and pH_i values were calculated using the fitted parameter values. Calibration curves for cells maintained at 37°C and for cells heated at 45°C for 10 min, then either held at 45°C or returned to 37°C for measurements, were similar for all treatment groups (Fig. 1). In a related experiment, cells were heated in Hanks' solution buffered with 25 mM HCO_3^--5% CO_2, and pH_i was measured in the presence of HCO_3^--CO_2.

To measure the initial alkalinization rate (recovery from acidification) of acid-loaded cells, cells were incubated for 10 s–15 min in Hanks' solution containing NH_4Cl (5–70 mM) with NaCl (75–140 mM) added to maintain isosmolarity. After NH_4Cl loading, cells were resuspended in NH_4Cl and Na^+-free Hanks' solution for 2 min to set pH_i to different levels. Recovery from acidification was measured as soon as the cells were resuspended in Na^+-containing Hanks' solution. The initial rate of recovery was determined by measuring the

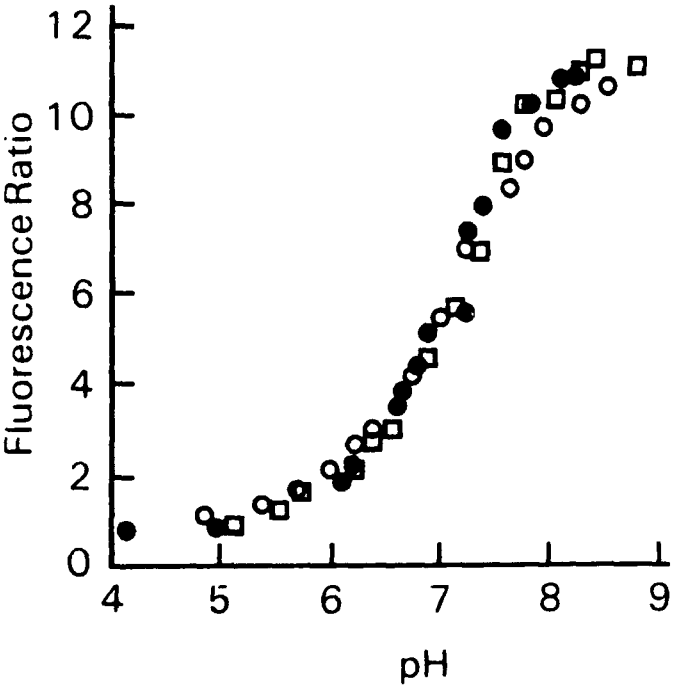

FIG. 1. Relationship of fluorescence ratio to pH. Cells were heated at 45°C for 10 min, then returned to 37°C (○) or held at 45°C (□) in K^+ Hanks' solution before taking the fluorescence measurement (n = 15). Non-heat-treated cells were held at 37°C (●).

slope of a fitted straight line to the initial 30 s of the fluorescence trace.

Measurement of H^+ extrusion. The rate of H^+ extrusion from suspended cells is derived from measurement of external pH according to the method of Grinstein et al. (17). Continuously shaken cell suspensions (4×10^6 cells) were maintained in lightly buffered (0.5 mM HEPES) Hanks' solution at 37 or 45°C for 10 min, and pH_o was then measured at 37°C every 2 min for 10 min using a Corning model 125 or 250 pH meter. The pH in the Hanks' solution was in the range of 7.1–7.3. H^+ extrusion was calculated by multiplying the change in pH_o between 0 and 10 min by the buffering power of the medium, which was determined by titration. Results are expressed in units of nmoles H^+ extruded per 10^6 cells per minute.

Measurement of intracellular buffering power. Cellular buffering power was measured according to methods of Boron (2) and Ng and Dudley (31). pH_i of cells (1×10^6 cells/ml) was set by exposing the cells to nigericin (2 mM) for 5 min in K^+ Hanks' solution having pH ranging from 6.5 to 7.8. After incubation, cells were centrifuged, washed, and resuspended in 145 mM K^+ Hanks' solution containing 1% nonesterified fatty acid-free bovine serum albumin, which scavenged nigericin from the cell membrane. Aliquots of NH_4Cl (1–8 mM) were then added, and alkalinization of the cells was measured. Buffering power was calculated from the equation

$$\text{Buffering power} = \Delta[NH_4^+]/\Delta pH_i$$
$$= [NH_3]_i \times 10^{(pK_a - pH_i)}/\Delta pH_i$$

where pH_i is the value following addition of NH_4Cl. The pK_a of NH_4^+ is 8.89 at 37°C and is assumed to be unchanged inside the cell. $[NH_3]_i$ is assumed to be equal to $[NH_3]_o$, which is calculated from the Henderson-Hasselbach equation.

Intracellular Na^+ measurements. Cells (2×10^7) were centrifuged through Versilube F50 silicone fluid (General Electric) using a Beckman microfuge. The aqueous phase and the oil phase were gently aspirated and discarded. The cell pellet was resuspended in 1 ml deionized distilled water for 1 h and sonicated for 5 min to completely lyse the cells. Lysates were centrifuged at 1,500 *g* for 10 min to remove particulate debris, and the supernatant was collected. The Na^+ content of the supernatant was determined by emission flame photometry (Instrumentation Laboratory model 09430–12). To determine cell water content, the tip of the polypropylene centrifuge tube containing the cell pellet was weighed before and after drying at 80°C for 72 h, with the difference representing the wet weight of the cell. Wet weight was found to be 33 $\mu l/10^6$ cells. Values for intracellular Na^+ and water content were corrected for extracellular fluid trapped in the cell pellet after centrifugation through the oil cushion using [^{3}H]sucrose (Du Pont-New England Nuclear, Boston, MA) as an extracellular marker (25).

ATP measurements. Cellular ATP was determined after extracting 1×10^6 cells/sample with ATP-releasing reagent (a detergent containing NaOH, product no. FL-SAR, Sigma Chemical, St. Louis, MO) for 15 min at 0°C. The extract was assayed for ATP contents by using the luciferase-luciferin assay (kit FL-ASC, Sigma) with a luminometer (Picolite model 6500).

Solutions. Hanks' solution contained (in mM) 148 NaCl, 4.6 KCl, 1.2 $MgCl_2$, 1.6 $CaCl_2$, and 10 HEPES (pH 7.26 at 37°C). The pH of Na^+ Hanks' solution heated to 45°C is 7.11. In Na^+-free Hanks' buffer, NaCl was substituted by equimolar concentrations of either choline chloride, *N*-methyl-(+)-glucamine chloride (NMG), or D-(+)-glucosamine chloride. In bicarbonate-buffered Na^+ Hanks' solution, HEPES was replaced by 25 mM $NaHCO_3$ and bubbled with 5% CO_2 (pH 7.26 at 37°C).

Statistical analysis. All data are expressed as means ± SE. Nonlinear least-squares fits to the data were carried out using the RS1 statistical package (BBN Software, Cambridge, MA). Analysis of variance (ANOVA), Studentized range test, Bonferroni's inequality, Student's *t* test, linear regression, and Mann-Whitney test were used for comparison of groups (40).

Chemicals. BCECF/AM, nigericin, and valinomycin were purchased from Molecular Probes (Eugene, OR). Other chemicals used in this study were sodium azide, 2-deoxy-D-glucose, trypan blue, ethidium bromide, acridine orange, amiloride, bovine serum albumin (fatty acid and globulin-free, product no. A-0281, Sigma), choline chloride, *N*-methyl-(+)-glucamine, and D-(+)-glucosamine chloride (Sigma).

RESULTS

Heat-induced intracellular acidification in bicarbonate-free Na^+ Hanks' solution. The resting pH_i of A-431 cells at 37°C in Hanks' solution (pH_o 7.26) is 7.23 ± 0.02 (n = 24). Cells subjected to temperatures >37°C for 10 min exhibit an intracellular acidification that is temperature dependent, and maximal acidification (−0.25 pH units) is observed at 50°C (Fig. 2). Further acidification is not observed >50°C (data not shown). The degree of acidification also depends on the duration of exposure to heat (Fig. 3). The pH_i of cells exposed to 45°C for 10 s–30 min decreases from 7.20 ± 0.05 to 6.99 ± 0.06. In this study heat treatment decreased the pH_o from 7.26 to 7.11, but

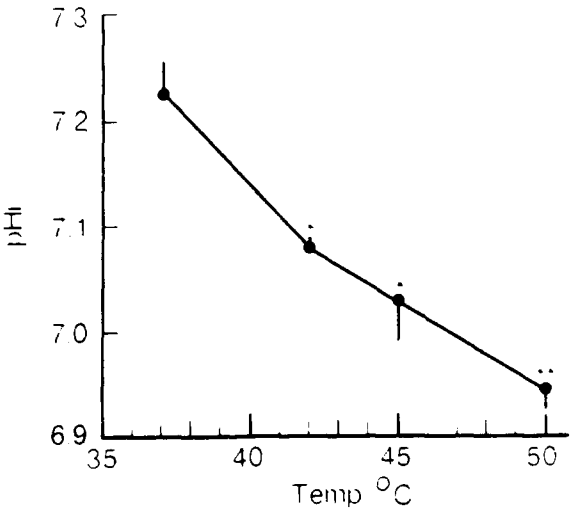

FIG. 2. Effect of temperature on intracellular pH (pH_i). Cells were exposed to 37, 42, 45, or 50°C for 10 min, then pH_i was measured (n = 3–22). * $P < 0.05$ vs. 37°C, ** $P < 0.05$ vs. 37 and 42°C. 1-way ANOVA and Bonferroni's inequality test.

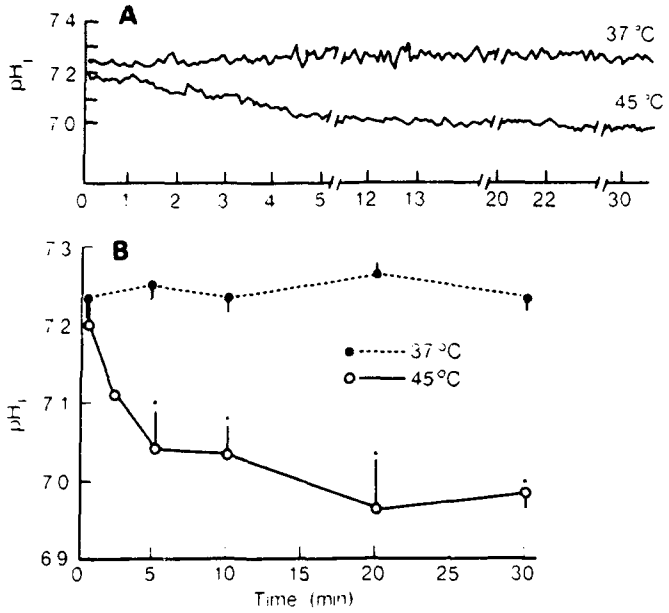

FIG. 3. Effect of duration of heating on pH$_i$. Cells were exposed to 45°C for different times (n = 3–17). *A*: fluorometer tracing of cells exposed to 45°C. *B*: pH$_i$ decreases with duration of heat exposure. *P < 0.05 vs. control, 2-way ANOVA and Bonferroni's inequality test.

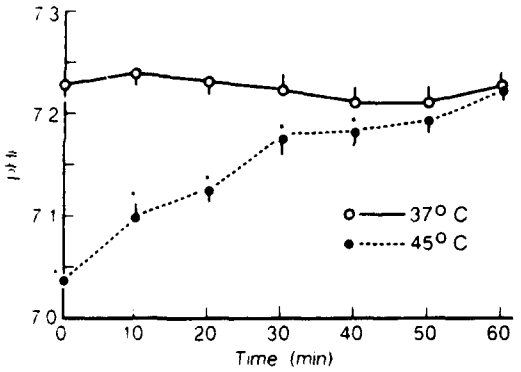

FIG. 4. Recovery after acidification induced by exposure to 45°C for 10 min (n = 4–6). *P < 0.05 vs. 37°C, 2-way ANOVA and Bonferroni's inequality test.

the degree of acidification is similar even when pH$_o$ is maintained at 7.26. Although heat treatment under these conditions does not induce acidification below pH$_i$ of ~6.95, this value did not represent an absolute limit. That is, cells that were preacidified to 6.9 ± 0.01 (n = 3) by a prepulse of NH_4Cl and maintained in Na^+-free Hanks' solution during heat treatment (45°C for 10 min) still show a decrease in pH$_i$ to 6.78 ± 0.04 (n = 4). Furthermore, cells exposed to K^+ Hanks' solution (145 mM KCl) have a lower than normal resting pH$_i$ of 7.0 ± 0.04 (n = 7) but still acidify in response to heat treatment to 6.8 ± 0.04 (n = 4).

Recovery from the acidification induced by heat treatment was examined in cells exposed to 45°C for 10 min, then incubated at 37°C for up to 1 h. Figure 4 demonstrates that the pH$_i$ in heat-treated cells returns to control (unheated) levels after 1 h. Amiloride prevents the recovery from heat-induced acidification. Cells that had been heat treated (45°C for 10 min), then incubated at 37°C for 1 h in the presence of amiloride (100 μM), have a pH$_i$ of 7.07 ± 0.02 (n = 4). This value is not statistically different from the pH$_i$ of cells immediately after heat treatment (7.03 ± 0.02, n = 16) but is significantly less than the pH$_i$ of heat-treated cells allowed to recover in the absence of amiloride (7.22 ± 0.01, n = 4).

To assess whether the heat-induced pH$_i$ changes are related to cell death, cell viability was measured using several different methods. The data in Table 1 demonstrate that the viability of heat-treated cells (45°C for 5 or 10 min) assessed by trypan blue exclusion and ethidium bromide-acridine orange staining is not different from that of unheated cells. Cells exposed to 45°C for 30 min exhibit a small (5–10%) decrease in viability, which is confirmed by lactate dehydrogenase measurements. Because it is possible that the cells are viable immediately after heat treatment, but become nonfunctional later, the plating efficiency of heated cells was examined 24 h after heat treatment. Cells heated at 45°C for 10 or 30 min were replated on culture dishes immediately after heat treatment, and 24 h later the percentage of adherent cells in heat-treated groups was compared with that of adherent cells in the control groups. Exposure to 45°C for 10 min has little effect on plating efficiency, but a 30-min exposure to heat decreases the plating efficiency by 10%. Based on these results and the data on heat-induced acidification, heat treatment at 45°C for 10 min was selected for subsequent experiments.

Heat-induced intracellular acidification in HCO_3^--CO_2-buffered Hanks' solution. Cassel et al. (5) and Ganz et al. (14) have reported that stimulus-induced pH$_i$ changes can be attenuated in the presence of bicarbonate. To determine whether heat-induced intracellular acidification occurs in the presence of bicarbonate, we measured the heat-induced pH$_i$ changes in cells suspended in Na^+ Hanks' solution buffered with 5% CO_2–25 mM HCO_3^- (pH$_o$ 7.26). As noted by Cassel et al. (5), the resting pH$_i$ of A-431 cells in the presence of bicarbonate (7.42 ± 0.03, n = 13) is higher than that in Na^+ Hanks' solution (7.23 ± 0.02, n = 24). Surprisingly, amiloride (100 μM) decreases the pH$_i$ of cells in bicarbonate Hanks' solution to 7.29 ± 0.04, supporting the view that the Na^+-H^+ exchanger contributes to the resting pH$_i$ even in the presence of bicarbonate. However, even in the presence of bicarbonate, pH$_i$ declines from 7.42 ± 0.03 (n = 13) to 7.22 ± 0.04 (n = 9) following heat treatment for 10 min at 45°C. This heat-induced intracellular acidification is

TABLE 1. *Cell viability following thermal injury*

	Viability, %			
	Trypan blue	EB/AO	LDH	Plating efficiency
Control	100	100	9.3±0.8	94±1
Heated				
5 Min	99±1	*	8.8±0.5	*
10 Min	97±2	97±0.4	11.2±1.6	90±1
30 Min	90±4†	95±1†	23.4±3.7†	84±2†

Values are means ± SE expressed as percentage of total cell population (n = 3–6 experiments). Cells were heated at 45°C for indicated periods of time, then returned to 37°C and assayed. EB/AO, ethidium bromide-acridine orange; lactate dehydrogenase (LDH) measurements represent percentage of total cellular LDH released into supernatant. * Not done. † P < 0.05 vs. control, determined by Student's t test.

not observed when cells are heat treated in the presence of amiloride (100 μM) (pH_i = 7.29 ± 0.04 at 37°C, 7.25 ± 0.05 at 45°C; n = 7 for both groups). Thus heat-induced acidification occurs under physiological buffering conditions.

Because the steady-state pH_i of A-431 cells in the absence or presence of bicarbonate is well above electrochemical equilibrium (~6.2 at 37°C assuming pH_o 7.25 and membrane potential = −65 mV), energy-dependent processes must be involved in maintaining steady-state pH_i. Inhibition of any of these by heat treatment can result in intracellular acidification. The rest of our study is focused on determining the effect of heat treatment on the functioning of one transport system involved in regulating pH_i, Na^+-H^+ exchange, and metabolism, which supplies the energy necessary for maintaining ionic gradients. These experiments were conducted in bicarbonate-free Na^+ Hanks' solution to eliminate the effect of bicarbonate-dependent process on pH_i.

Characterization of Na^+-H^+ exchange. In cells in which Na^+-H^+ exchange contributes to resting pH_i, an inhibition of this exchange could lead to intracellular acidification. Na^+-H^+ exchanger activity was determined in resting cells by measuring the effect of amiloride on resting pH_i. Amiloride (100 μM) causes resting pH_i to decrease from 7.23 ± 0.02 (n = 24) to 7.08 ± 0.01 (n = 5) (Fig. 5, *top tracing*). As will be discussed later, amiloride does not reduce the resting pH_i of heated cells (Fig. 5, *bottom tracing*).

Recovery from an imposed acid load is also mediated by Na^+-H^+ exchange in these cells. Acid-loaded cells (prepulsed with 40 mM NH_4Cl Hanks' solution for 15 min) have a pH_i of 6.69 ± 0.02 (n = 22) in Na^+-free (NMG) Hanks' solution, but their pH_i returns to 7.2 within 4 min after being placed in Na^+ Hanks' solution (Fig. 6*A*, *inset*). Recovery is both $[Na^+]_o$ dependent and inhibited by amiloride. The initial rate of recovery is inhibited 96% in Na^+-free solution. Increasing $[Na^+]_o$ from 0 to 145 mM increases the rate of recovery, with an apparent Michaelis constant (K_m) for extracellular Na^+ of 38 ± 9 mM (Fig. 6*A*). Amiloride (100 μM) inhibits the initial rate of recovery from acidification in Na^+ Hanks' solution by 96%. Inhibition is concentration dependent with an apparent mean affinity constant ($K_{\frac{1}{2}}$) for amiloride of 11 ± 3 μM (Fig. 6*B*). Because the rate of recovery is inhibited by 96% in both Na^+-free and amiloride-containing solutions, Na^+-H^+ exchange appears to be the primary process responsible for recovery from an acid load in these cells in bicarbonate-free buffer. Thus, if heat treatment inhibits the activity of the Na^+-H^+ exchanger, then the rate of recovery from an acid load should be inhibited in heated cells.

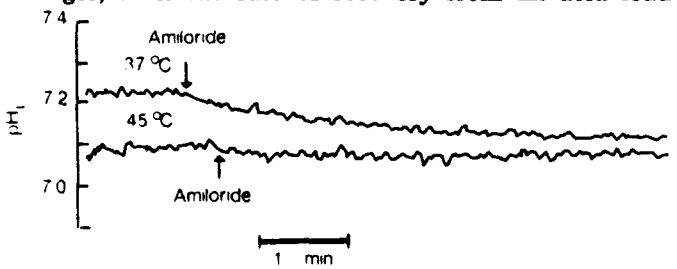

FIG. 5. Fluorometer tracings of effect of amiloride (100 μM) on pH_i of control (37°C) and heat-treated (45°C) cells.

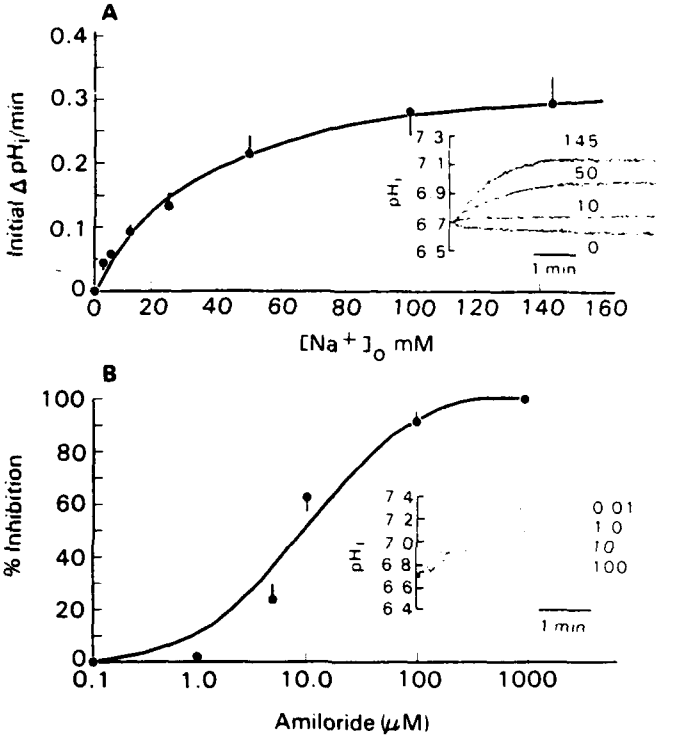

FIG. 6. Evidence for Na^+-H^+ exchange in A-431 cells. Cells were loaded with NH_4Cl (40 mM) for 15 min, placed in Na^+-free (NMG) medium for 2 min, then resuspended in buffer containing different concentrations of Na^+. *A*: rate of alkalinization following acid loading is dependent on $[Na^+]_o$ (n = 3–4). Na^+-insensitive portion of recovery was substracted from these values. Apparent K_m for Na^+ is 38 ± 9 mM. *Inset*: fluorometer tracings of time required for acid-loaded cells to alkalinize in the presence of several Na^+ concentrations. No recovery is observed in Na^+-free Hanks' solution. *B*: amiloride blocks recovery from acid loading (n = 3–4). Apparent $K_{\frac{1}{2}}$ for amiloride is 11 ± 3 μM. *Inset*: fluorometer tracings with different concentrations of amiloride (in μM).

Heat-induced inhibition of recovery from acidification. The effect of heat treatment (45°C for 10 min) on the rate of recovery from an acid load is shown in Fig. 7. After heat treatment, cells were returned to 37°C for either 2 min or 1 or 2 h, then acid loaded and allowed to recover. The fluorescence tracings from these experiments are shown in Fig. 7*A*. Cells allowed to recover for either 2 min or 1 h before acid loading show a reduced rate of recovery. In cells allowed to recover for 2 h before they are acid loaded, the rate of recovery from acid loading is not different from that of control cells.

In A-431 cells, as in other cell types, the initial rate of recovery from acidification is proportional to the magnitude of acidification (4, 39). Figure 7*B* shows that heat treatment changes the slope of the pH_i vs. recovery rate curve. Cells that had been heat treated and allowed to recover for either 2 min or 1 or 2 h were acid loaded to different pH_i values, and the initial rate of recovery was measured as a function of pH_i. (It should be noted that the cells allowed to recover from heat treatment for 2 min were acid loaded to pH_i values of only 6.73 or greater because acid loading below that value required >5-min incubation periods.) The dependence of the rate of recovery on pH_i for unheated and heat-treated cells allowed

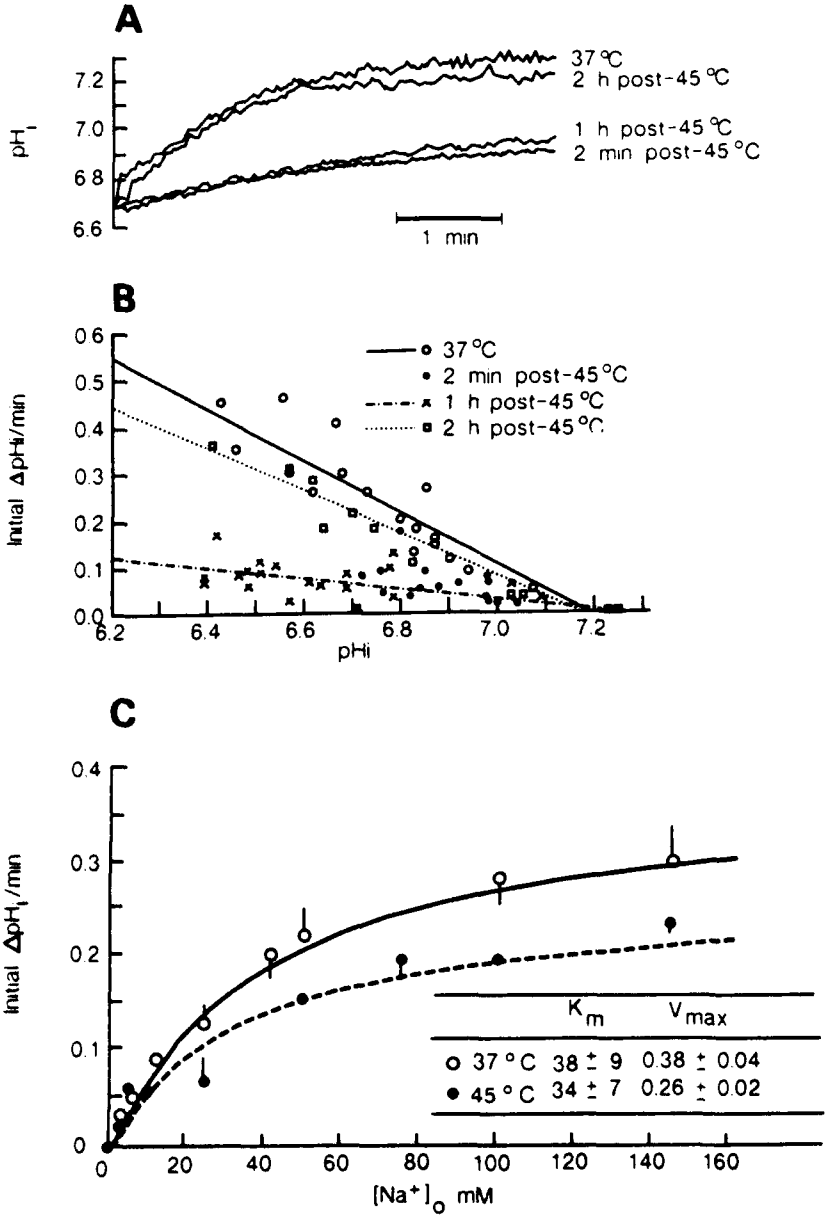

FIG. 7. Effect of heat treatment on rate of alkalinization of acid-loaded cells. Cells were heated at 45°C for 10 min, acid-loaded by pulsing with NH_4Cl (5–70 mM) immediately or 1 or 2 h after heat treatment, then placed in Na^+-free (NMG) media for 2 min to set their pH_i, then resuspended in Na^+ Hanks' solution for measurements. *A*: fluorometer tracings of effect of acid loading the cells at different times after heat treatment. *B*: relationship of initial rate of alkalinization to the level of acidification following acid loading of unheated cells (n = 18) and cells loaded 2 min (n = 15), 1 h (n = 20), or 2 h (n = 12) after heat. Correlation coefficients of the linear regression lines are 0.92, 0.97, and 0.88 for unheated, 1 h postheat, and 2 h postheat, respectively. *C*: initial rates of alkalinization from an acid load with different $[Na^+]_o$ in heated cells 1-h postheat treatment. Inserted table represents the V_{max} and apparent K_m for external Na^+ in unheated and heat-treated cells 1 h after heating.

to recover for 2 h is the same. However, the rate of recovery for heat-treated cells either 2 min or 1 h after heat treatment is significantly reduced over a wide range of pH_i. The x-intercept of the line for the data obtained 1 h after thermal injury is not significantly different from the x-intercept of the line through the data obtained from unheated cells, indicating no shift in the "set point" of the curve. This is consistent with the observation that the resting pH_i of heat-treated cells is normal after 1 h.

Because the apparent inhibition of Na^+-H^+ activity could be due to a change in maximal initial alkalinization rate (V_{max}) of the exchanger, or to changes in the affinity of the exchanger for extracellular Na^+ or intracellular H^+, the apparent affinity for extracellular Na^+ was evaluated in cells that were heat treated at 45°C for 10 min, then returned to 37°C for 1 h. Figure 7*C* shows that the apparent K_m for extracellular Na^+ in heat-treated cells is not statistically different from that of unheated cells, whereas the apparent V_{max} is ~40% slower than that of unheated cells. The apparent affinity for H_i^+ was not calculated.

Although our data are consistent with the idea that Na^+-H^+ exchange has been inhibited, changes in either cellular buffering capacity or the Na^+ gradient could also produce these effects. Cellular buffering power in unheated and heat-treated cells 1 h after heating was measured (Fig. 8). As has been shown in many other cell types (2, 31, 36), buffering power values vary with pH_i. In A-431 cells, buffering power remains constant in the pH_i range 7.2–7.8 in both groups but is inversely proportional to pH_i below pH_i 7.2. However, heat treatment does not change the buffering power in these cells. Furthermore, the $[Na^+]_i$ in both groups is not statistically different (unheated: 27 ± 3 mmol/l cell water, n = 7; heat treated: 29 ± 9 mmol/l cell water, n = 7), indicating that heat-induced intracellular acidification is not due to a decrease in the Na^+ gradient. These results are similar to the

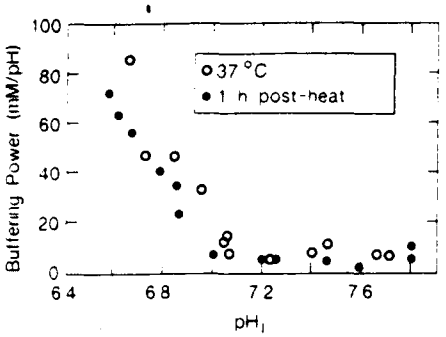

FIG. 8. Relationship of cellular buffering power to pH$_i$ in cells suspended in nominally bicarbonate-free buffer exposed to 37 or 45°C for 10 min (n = 18–22).

TABLE 2. *Removal of Na^+ reduces magnitude of heat-induced acidification*

	pH$_i$		
	37°C	45°C	Decrease
Na^+	7.23±0.02	7.03±0.03	0.20±0.02
Choline	6.93±0.03	6.83±0.03	0.10±0.03*
D-(+)-Glucosamine	7.08±0.02	6.95±0.02	0.13±0.02*
N-methyl-(+)-glucamine	7.52±0.01	7.37±0.02	0.15±0.02*

Values are means ± SE (n = 3–24 experiments). Cells were heated in specified Hanks' solution at 45°C for 10 min. pH$_i$, intracellular pH. * $P < 0.05$ vs. Na^+ Hanks' solution, Student's t test.

observation that intracellular Na^+ does not change in mouse mastocytoma p815 cells exposed to 43°C for 1 h (48).

Magnitude of heat-induced acidification is reduced under conditions that inhibit the Na^+-H^+ exchanger. The relationship between heat-induced intracellular acidification and the activity of the Na^+-H^+ exchanger was determined by measuring the magnitude of heat-induced acidification in the presence of amiloride. If heat-induced acidification were due solely to an inhibition of Na^+-H^+ exchange, then amiloride-treated cells should not be acidified upon heating. The pH$_i$ of amiloride-treated cells is 7.08 ± 0.03 (n = 4), while the pH$_i$ of cells heated at 45°C for 10 min in amiloride-containing Na^+ Hanks' solution is 6.990 ± 0.006 (n = 5). The magnitude of the heat-induced pH$_i$ change is only 50% of the heat-induced pH$_i$ change in the absence of amiloride. Conversely, when amiloride (100 μM) is added to heat-treated cells (7.03 ± 0.02, n = 16) the pH$_i$ is not reduced further (6.99 ± 0.04; n = 6; Fig. 5, *bottom tracing*).

Experiments were also conducted in Na^+-free Hanks' solution in which the activity of the Na^+-H^+ exchanger was inhibited by removing extracellular Na^+. Heat treatment produces additional intracellular acidification in the absence of extracellular Na^+, although the degree of acidification is reduced (Table 2). When either choline or D-(+)-glucosamine was used as a Na^+ substitute, the resting pH$_i$ decreases, as it did in the presence of amiloride, presumably because the Na^+-H^+ exchanger is inhibited. The heat-induced acidification is still observed, but its magnitude is 50% of that in Na^+-containing Hanks' solution. When NMG is used as a Na^+ substitute, the resting pH$_i$ becomes more alkaline (7.52 ± 0.01, n = 3), probably as a result of the entry of this weak base into the cell (39). Heat-induced acidification is also observed in this solution, although its magnitude is only 25% of that of the control. The data obtained with Na^+ substitutes together with the amiloride data indicate that an inhibition of Na^+-H^+ exchange accounts for ~50% of the heat-induced acidification.

Heat-induced inhibition of steady-state H^+ efflux. To further test the hypothesis that Na^+-H^+ exchange is inhibited by heat treatment, steady-state H^+ efflux, with an without amiloride or Na^+, was measured by monitoring the external pH of suspended cells in lightly buffered (0.5 mM HEPES) Na^+ Hanks' solution. This experiment also allows us to determine whether heat-induced intracellular acidification is the result of an increased uptake of external H^+. The results in Table 3 show that a suspension of control cells (Na^+ Hanks', 37°C) extrudes H^+ and that the rate of acid extrusion is reduced 80% in the presence of amiloride (100 μM) or in Na^+-free Hanks' solution, presumably due to inhibition of Na^+-H^+ exchange. In heated-treated cells, a decreased rate of acidification of the medium is observed, consistent with the hypothesis that Na^+-H^+ exchange is inhibited by heat treatment, and also consistent with the hypothesis that intracellular acidification is not a result of H^+ uptake. The rate of H^+ efflux recovers by ~50% at 1 h after heating and completely recovers at 2 h after heating. The time course of recovery is similar to that previously characterized for recovery of Na^+-H^+ exchange as shown in Fig. 7. Amiloride (100 μM) given immediately after heat treatment blocks the recovery of H^+ efflux (Table 3). Taken together, the measurements of pH$_i$ and H^+ efflux indicate that heat-induced intracellular acidification is not the result of uptake of extracellular H^+, but rather a result of a reduced rate of acid extrusion, most probably via Na^+-H^+ exchange.

Heat-induced metabolic inhibition. It is known that metabolic inhibition can produce an intracellular acidification (3, 10, 32) and a reduction of intracellular ATP levels (20, 50). A previous study on A-431 cells (4) has demonstrated that reducing the cellular ATP content (to levels that were ~8% of control levels) inhibits Na^+-H^+ exchange by modulating an intracellular H^+-dependent regulatory mechanism. Therefore, to investigate further the mechanisms underlying the heat-induced acidification, experiments were performed to determine whether heat treatment inhibits ATP production in A-431 cells, resulting in an inhibition of Na^+-H^+ exchanger activity.

TABLE 3. *Heat treatment reduces steady-state H^+ efflux*

	H^+ Efflux, nmol·10^6 cells^{-1}·min^{-1}			
	37°C	Time after heat treatment		
		30 s	1 h	2 h
NMG	0.52±0.001	0.63±0.01	0.63±0.02	0.41±0.04
Na^+	2.27±0.13‡	0.26±0.01*	1.26±0.30†	1.92±0.40‡
Na^+ + amiloride	0.51±0.02§	0.25±0.12	0.49±0.03§	0.28±0.06§

Values are means ± SE (n = 3–5 experiments). Cells were exposed to 45°C for 10 min and then returned to 37°C to determine external pH in the medium lightly buffered with 0.5 mM HEPES. *N*-methyl-(+)-glucamine (NMG) replaced Na^+ in Na^+-free buffer. * $P < 0.05$ vs. Na^+ at 37°C and 1 and 2 h postheating. † $P < 0.05$ vs. Na^+ buffer at 37°C and 30 s postheating. ‡ $P < 0.05$ vs. NMG at 37°C and Na^+ buffer 30 s postheating. § $P < 0.05$ vs. Na^+ buffer.

Heat treatment (45°C for 10 min) reduces cellular ATP content, with longer exposures leading to further decreases in ATP levels (Fig. 9*A*). When heat-treated cells are returned to 37°C in Hanks' solution (containing no nutrients), ATP levels continue to fall, reaching a minimum 20 min after heating. The ATP content then returns slowly to control levels (that also decreased) 90 min after heat treatment (Fig. 9*B*). At 120 min the ATP content in both control and heat-treated cells is reduced by 46 ± 1 and 47 ± 2%, respectively. Nevertheless, as shown in Fig. 7*A*, and Table 3, the rate of recovery from acidification of cells and H^+ efflux 2 h after heat treatment is normal, and the resting pH$_i$ of both unheated and heat-treated cells is similar to control cells at the beginning of the experiment. These findings indicate that Na^+-H^+ exchange in A-431 cells is unaffected by a reduction of >50% in intracellular ATP content.

The effect of heat on anaerobic glycolysis was studied by measuring lactic acid production. Lactic acid is reduced by 25 ± 5 and 42 ± 9% in cells exposed to 45°C for either 10 or 30 min, respectively (Fig. 10*A*). Thirty minutes after treatment (45°C, 10 min) the lactic acid content is still lower than controls but returns to normal levels 1 h after heat treatment (Fig. 10*B*).

Effect of metabolic inhibitors on heat-induced acidification. Because only 50% of the heat-induced acidification can be ascribed to inhibition of Na^+-H^+ exchange,

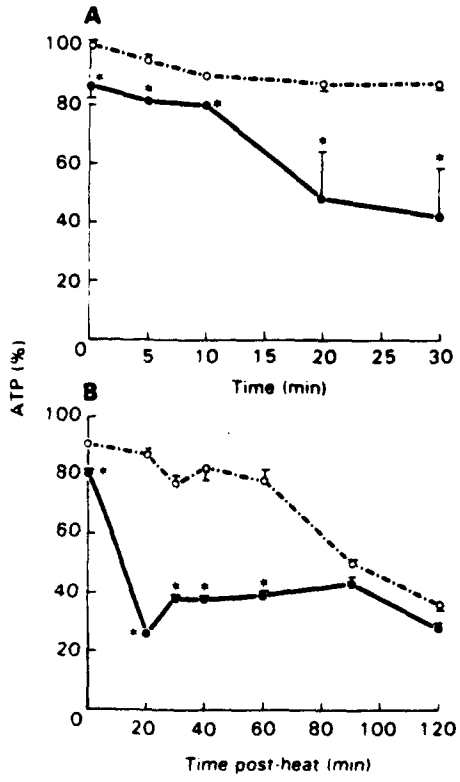

FIG. 9. Effects of heat treatment on ATP content. *A*: heat reduces cellular ATP content. Cells were heated at 45°C for 10 s or 5, 10, 20 or 30 min (n = 4). Data are expressed as percentage of control cell ATP content at the start of experiment (4.160 ± 0.003 nmol/10^6 cells). *P < 0.05 vs. 37°C. *B*: recovery of cellular ATP content at various times after heating at 45°C for 10 min (n = 3–4). Values are expressed as percentage of control cell ATP content at the start of experiment. * P < 0.05 vs. 37°C, 2-way ANOVA and Bonferroni's inequality test. ●, Heated cells; ○, unheated cells.

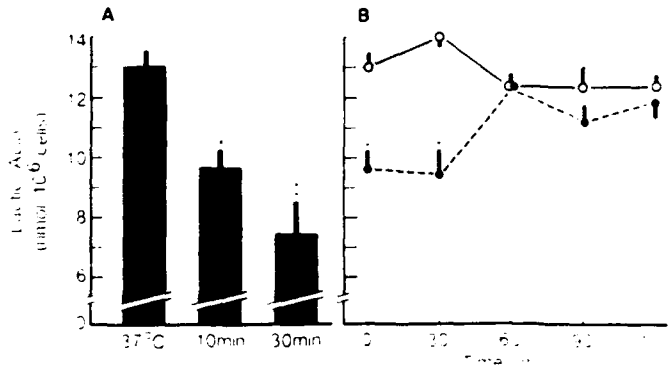

FIG. 10. Role of metabolism in heat-induced acidification. *A*: heat reduces lactic acid content. Cells were heated at 45°C for 10 or 30 min (n = 4–9). * P < 0.05 vs. 37°C, 1-way ANOVA and Bonferroni's inequality test. **P < 0.05 vs. 37°C for 10 min and 45°C for 10 min. *B*: recovery of lactic acid content at various times after heating at 45°C for 10 min (n = 4–9). * P < 0.05 vs. 0 min postheat, 2-way ANOVA and Bonferroni's inequality test. ●, Heated cells; ○, unheated cells.

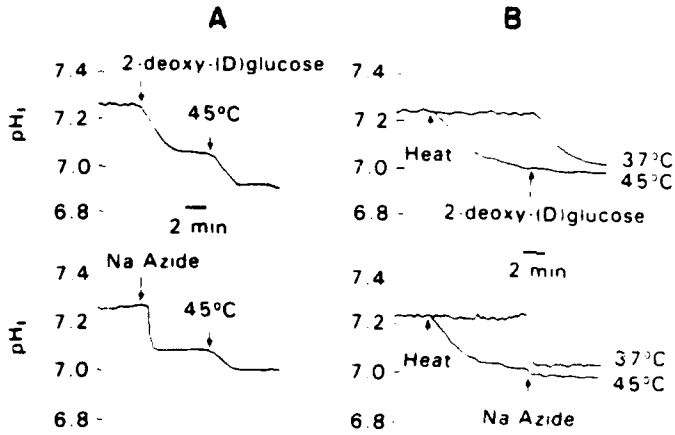

FIG. 11. Effects of 2-deoxy-D-glucose or sodium azide, given before or after heat treatment, on heat-induced intracellular acidification. *A*: fluorometer tracings of cells treated with heat after administration of 2-deoxy-D-glucose (10 mM) or sodium azide (10 mM). *B*: fluorometer tracings of cells exposed to 2-deoxy-D-glucose (10 mM) or sodium azide (10 mM) after heat treatment.

and because metabolic activity is reduced by heat treatment, we tested the hypothesis that the remaining inhibition could be due to metabolic inhibition. We examined the effects of 2-DG (10 mM), a glucose antagonist that inhibits glycolysis, and sodium azide (10 mM), an inhibitor of oxidative phosphorylation, on pH$_i$. Both agents induce an intracellular acidification that is maintained for 10 min. The maximal acidification induced by 2-DG (7.046 ± 0.020, n = 3) occurs within 5 min, while the maximal acidification induced by sodium azide (7.080 ± 0.037, n = 4) is immediate (Fig. 11*A*). Addition of either inhibitor to cells 5 min before heat treatment reduces the magnitude of intracellular acidification (controls: 0.200 ± 0.036 units; with 2-DG: 0.094 ± 0.029 unit; with sodium azide: 0.06 ± 0.044 unit, n = 3–24; P < 0.05). In contrast, when cells are heated before addition of 2-DG no further acidification is noted (Fig. 11*B*). Addition of sodium azide further acidifies heat-treated cells only slightly. The results support the view that the inhibition of glycolysis and oxidative phosphorylation contributes to heat-induced acidification.

In these studies the cellular ATP content of cells treated with 2-DG or sodium azide in Na^+ Hanks' solution for 5 min are 72 ± 2% (n = 6) and 37 ± 10% (n = 6) of controls, respectively (controls: 3.94 ± 0.074 nmol/10^6 cells, n = 6). To determine whether metabolic inhibition by 2-DG or sodium azide acidifies the cells by lowering ATP content, thereby inhibiting Na^+-H^+ exchange, cells were placed in either Na^+-free Hanks' solution for 5 min or Hanks' solution containing amiloride (1 mM) for 10 min to block the Na^+-H^+ exchanger. Then, 2-DG (10 mM) or sodium azide (10 mM) was applied to the cells for 5 min. Under these conditions, intracellular acidification is still observed even though Na^+-H^+ exchange is blocked (in Na^+-free Hanks' solution, 2-DG: 0.07 ± 0.03 units; sodium azide: 0.126 ± 0.07 units, n = 4; in amiloride Hanks' solution, 2-DG: 0.09 ± 0.05 units; sodium azide: 0.14 ± 0.03 units, n = 4).

The possibility that the heat-induced acidification is due to the inhibition of both Na^+-H^+ exchange and metabolism was tested by treating cells with both amiloride and 2-DG for 5 min, and then exposing these cells to 45°C for 10 min. Treatment with both amiloride (100 μM) and 2-DG (10 mM) induces a maximal acidification (7.05 ± 0.02, n = 4) within 5 min that is maintained during the next 10 min. Figure 12 shows that heat does not induce further acidification in cells treated with both amiloride and 2-DG (7.00 ± 0.02, n = 5; $P > 0.05$).

DISCUSSION

This study demonstrates that hyperthermia produces an intracellular acidification (~0.2 pH units) in human epidermoid carcinoma A-431 cells that is temperature and time dependent. Acidification is not related to cell death, because cells that are heated at 45°C for 10 min remain viable, have the same plating efficiency as unheated cells, and recover their pH$_i$ to normal levels 1 h after heat treatment. Similar levels of heat-induced acidification have been noted in nonepithelial cells. For example, mouse mastocytoma cells acidify by 0.35 pH units following heat treatment for 30 min at 43°C (48), and mouse soleus muscle acidifies by 0.16 pH units when heated from 28 to 37°C (1). Likewise, *Drosophila* salivary gland cells acidify by 0.47 pH units when heated from 25 to 35°C (9), and yeast cells acidify by 0.65 pH units when heated from 23 to 40°C (46).

To investigate the mechanisms underlying the heat-induced acidification, we examined the properties of the Na^+-H^+ exchanger in control and thermally injured A-431 cells. Our findings in unheated A-431 cells in suspension are similar to those of Rothenberg et al. (37, 38), who previously characterized Na^+-H^+ exchange in adherent A-431 cells. In agreement with our results, they reported a resting pH$_i$ of 7.3 ± 0.1 at 37°C (pH$_o$ 7.2) that declines in the presence of amiloride, indicating that the Na^+-H^+ exchanger is necessary to maintain resting pH$_i$. A similar role for the Na^+-H^+ exchanger has been reported in principal and intercalated cells of rabbit cortical collecting tubule (45). Also in agreement with our results, Rothenberg et al. (37, 38) found that a significant fraction (at least 50%) of the recovery from acidification under nominally bicarbonate-free conditions is mediated by Na^+-H^+ exchange. The amiloride sensitivity of Na^+-H^+ exchange (11 ± 3 μM) in our studies is similar to the 4 μM found by Zhuang et al. (49).

The rate of recovery from acid loading, probably mediated by Na^+-H^+ exchange, is markedly inhibited in heat-treated cells over a wide range of pH$_i$ values. In cells heat treated at 45°C for 10 min and returned to 37°C for 1 h, the V_{max} is ~40% slower than that of unheated cells. The apparent K_m for $[Na^+]_o$ is unchanged. Similar results have been reported in a preliminary study of EM T6 cells treated with heat at 45°C for 30 min (27). There are only a few reports of stimulus-induced inhibition of Na^+-H^+ exchange in other systems to be compared with our findings. Parathyroid hormone (PTH) inhibits Na^+-H^+ exchange in renal brush border vesicles (26) and in cultured kidney (OK) cells (22). Interestingly, in OK cells the PTH-induced inhibition results in a reduction of V_{max}, similar to our results with A-431 cells (Fig. 7*B*). Inhibition of Na^+-H^+ exchanger activity has also been induced in rat osteosarcoma cells by osmotic swelling (16). Other factors that can alter the activity of the Na^+-H^+ exchanger include growth factors and osmotic shrinkage, but these treatments result in stimulation of the Na^+-H^+ exchanger (17, 18).

Three observations support the view that the acidification produced by thermal injury is due in part to an inhibition of Na^+-H^+ exchange. First, the rate of recovery from acidification, which is mediated primarily by Na^+-H^+ exchange, is inhibited by heat treatment. This inhibition is not due to a reduction in the gradient of Na^+ or changes in buffering capacity. Second, acidification is reduced in Na^+-free solutions or in the presence of amiloride. Heat treatment of control cells leads to an acidification of ~0.2 pH units, whereas heat treatment of cells that are placed in Na^+-free or amiloride-containing solution produces an acidification of ~0.1 pH units, indicating that about half of the acidification induced by thermal injury is due to an inhibition of Na^+-H^+ exchange. Third, heat-treated cells demonstrate less steady-state H^+ efflux than do unheated cells, and recovery of the H^+ efflux occurs 2 h after heat treatment and is blocked by amiloride.

Previous studies on A-431 cells by Cassel et al. (4) have demonstrated that depletion of intracellular ATP (to <10% of control levels) inhibits Na^+-H^+ antiporter activity due to modulation of an intracellular H^+-dependent regulatory mechanism. In addition, in other cell

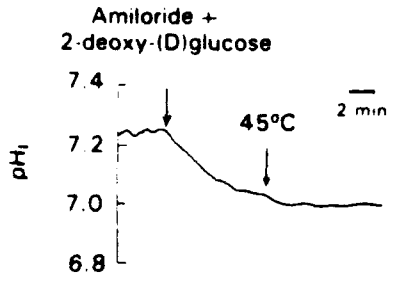

FIG. 12. Effects of 2-deoxy-D-glucose and amiloride, added before heat treatment, on heat-induced intracellular acidification. Fluorometer tracing of cells treated with 2-deoxy-D-glucose (10 mM) and amiloride (100 μM) together 5 min before heat treatment at 45°C for 10 min.

types, inhibition of glycolysis is associated with an intracellular acidification (3, 15). Therefore, we examined the possibility that the heat-induced acidification in A-431 cells was related to an inhibition of metabolism by measuring the levels of both ATP and lactic acid in unheated and heat-treated cells. Our data indicate that exposing cells to 45°C for 10 min inhibits glycolysis and reduces intracellular ATP by 23 ± 2 and 25 ± 5%, respectively. Under our experimental conditions (i.e., in the absence of nutrients), intracellular ATP contents of both heat-treated and control cells continue to decrease so that by 120 min after heat treatment the ATP content of both heated and unheated cells is 36% of the ATP content of unheated cells immediately before heat treatment. The pH_i for both groups of cells at this time is identical to the pH_i of the unheated cells at the start of the experiment. Furthermore, the rate of alkalinization after an acid load for both unheated and heat-treated cells 120 min after the start of the experiment (when their ATP content was reduced) is similar to unheated cells at the start of the experiment (Fig. 7*B*). These data indicate that the Na^+-H^+ exchanger functions normally even though the ATP content is reduced by 64%. Thus heat-induced inhibition of the Na^+-H^+ exchanger is not secondary to a heat-induced ATP reduction. The differences between our data and that of Cassel et al. (4) can be explained by the fact that ATP levels in our study are not depleted as fully as in their study.

To further investigate the role of metabolism in heat-induced acidification, we examined the effects of sodium azide and 2-DG on pH_i in unheated and heat-treated cells. (We also attempted to study the effects of 2,4-dinitrophenol, an inhibitor of oxidative phosphorylation, but its inherent flourescence interfered with the assay.) 2-DG substitutes for glucose but cannot be metabolized beyond the hexose monophosphate shunt. This reduces ATP production via anaerobic glycolysis and subsequently slows aerobic glycolysis and the electron transport chain due to the low level of pyruvate in cells (20, 50). In A-431 cells 2-DG (10 mM) produces an acidification but does not further acidify cells that have been heat treated, supporting the view that the acidification induced by 2-DG is mediated via pathway(s) in common with acidification induced by heat. However, another explanation of these results is possible. 2-DG might not be able to acidify cells further because other buffers (that are not heat sensitive) may maintain the pH_i when it is <6.9. This possibility is unlikely, since data on cells that have been acidified in high-K^+ Hanks' solution or by acid loading to set pH_i to ~6.9 indicates that acidification induced by heat treatment can occur below pH_i 6.9. Sodium azide, an inhibitor of oxidative phosphorylation (29), also produces an acidification but more rapidly than does 2-DG. As is shown in Fig. 11*B*, application of sodium azide to heat-treated cells results in a small additional acidification that is possibly due to sodium azide acting as a weak acid ($pK_a = 4.7$) (3, 36).

Several observations suggest that heat-induced acidification in bicarbonate-free medium is caused by at least two different mechanisms: an inhibition of Na^+-H^+ exchange and metabolic inhibition. First, heat-treated cells are not acidified further by the addition of metabolic inhibitors, but heat treatment after the addition of metabolic inhibitors still produced an acidification that is ~50% of that produced in the absence of metabolic inhibitors. Second, the possibility that the changes in pH_i produced by 2-DG and sodium azide are due to an effect on Na^+-H^+ exchange are negated by the observation that cells become acidified in Na^+-free or amiloride-containing buffer (to inhibit Na^+-H^+ exchange) after the addition of metabolic inhibitors. Finally, heat treatment fails to induce acidification in cells treated with both amiloride and 2-DG.

To determine the physiological relevance of these changes, we also investigated the effects of heat treatment on A-431 cells in presence of bicarbonate buffer. Because stimulus-induced pH_i changes are known to be attenuated in the presence of bicarbonate (5, 14), we were somewhat surprised that a heat-inducible acidification of ~0.2 pH units that can be inhibited by amiloride is still evident in the presence of bicarbonate. A similar heat-induced acidification in the presence of bicarbonate has been reported in *Drosophila* salivary gland cells (9). Although the possibility of the bicarbonate-related exchangers being affected by heat and contributing indirectly to acidification cannot be ruled out, it seems unlikely because heat-induced acidification in the presence of bicarbonate is blocked by amiloride. Further studies are required to dissect out the role of bicarbonate-dependent transport systems in pH_i regulation of unheated and heated A-431 cells.

In summary, this paper demonstrates that thermal injury produces an intracellular acidification in A-431 cells of 0.2 pH units under both nominally bicarbonate-free or bicarbonate-buffered solutions. In the absence of bicarbonate, the acidification is caused by at least two different events: inhibition of the activity of the Na^+-H^+ exchanger and metabolic inhibition. The change in pH_i following thermal injury may modulate other cell functions such as cell growth and may be one determinant of the sensitivity of tumor cells to hyperthermia (6, 24, 33).

We thank Spencer Green for his technical assistance, William Jackson for his assistance on statistical analysis, and Dr. David E. McClain for his discussion and editorial assistance.

This work was supported by the Armed Forces Radiobiology Research Institute, Defense Nuclear Agency, under work unit 00020. Views presented in this paper are those of the authors; no endorsement by the Defense Nuclear Agency has been given or should be inferred.

Address for reprint requests: J. G. Kiang, Dept. of Clinical Physiology, Div. of Medicine, Bldg. 40, Walter Reed Army Institute of Research, Washington, DC 20307-5100.

Received 1 August 1989; accepted in final form 13 July 1990.

REFERENCES

1. AICKIN, C. C., AND R. C. THOMAS. An investigation of the ionic mechanism of intracellular pH regulation in mouse soleus muscle fiber. *J. Physiol. Lond.* 267: 791–810, 1977.
2. BORON, W. F. Intracellular pH transients in giant barnacle muscle. *Am. J. Physiol.* 233 (*Cell Physiol.* 2): C61–C73, 1977.
3. BORON, W. F., AND P. DE WEER. Intracellular pH transients in squid giant axons caused by CO_2, NH_3, and metabolic inhibitors. *J. Gen. Physiol.* 67: 91–112, 1976.
4. CASSEL, D., M. KATZ, AND M. ROTMAN. Depletion of cellular ATP inhibits Na^+-H^+ antiport in cultured human cells. *J. Biol. Chem.* 261: 5460–5466, 1986.
5. CASSEL, D., B. WHITELEY, Y. X. ZHUANG, AND L. GLASER. Mito-

gen-independent activation of Na^+-H^+ exchange in human epidermoid carcinoma A-431 cells: regulation by medium osmolarity. *J. Cell. Physiol.* 122: 178–186, 1985.

6. CHU, G. L., AND W. C. DEWEY. The role of low intracellular or extracellular pH in sensitization to hyperthermia. *Radiat. Res.* 114: 154–167, 1988.
7. CHU, G. L., Z. WANG, AND W. DEWEY. The effect of hyperthermia on intracellular pH (pH_i) and the role of pH_i in low pH sensitization of hyperthermic killing (Abstract). *Proc. Annu. Meet. Radiat. Res. Soc. 37th Seattle WA 1989*, p. 105.
8. DICKENS, C. J., J. I. GILLESPIE, J. R. GREENWELL, AND P. HUTCHINSON. Relationship between intracellular pH (pH_i) and calcium (Ca^{2+}) in avian heat fibroblasts. *Exp. Cell Res.* 187: 39–46, 1990.
9. DRUMMOND, I. A. S., S. A. MCCLURE, M. POENIE, R. Y. TSIEN, AND R. A. STEINHARDT. Large changes in intracellular pH and calcium observed during heat shock are not responsible for the induction of heat shock proteins in *Drosophila melanogaster*. *Mol. Cell. Biol.* 6: 1767–1775, 1986.
10. EISNER, D. A., C. G. NICHOLS, S. C. O'NEILL, G. L. SMITH, AND M. VALDEOLMILLOS. The effects of metabolic inhibition on intracellular calcium and pH in isolated rat ventricular cells. *J. Physiol. Lond.* 411: 393–418, 1989.
11. ELLIS, D., AND R. C. THOMAS. Direct measurement of the intracellular pH of mammalian cardiac muscle. *J. Physiol. Lond.* 26: 755–771, 1976.
12. FERNANDEZ-POL, J. A. Epidermal growth factor receptor of A-431 cells. *J. Biol. Chem.* 260: 5003–5011, 1985.
13. FRESHNEY, R. I. *Culture of Animal Cells: A Manual of Basic Technique*. New York: Liss, 1983.
14. GANZ, M. B., G. BOYARDSKY, R. B. STERZEL, AND W. F. BORON. Arginine vasopressin enhances pH_i regulation in the presence of HCO_3^- by stimulating three acid-base transport systems. *Nature Lond.* 337: 648–651, 1989.
15. GEVERS, W. Generation of protons by metabolic processes in heart cells. *J. Mol. Cell Cardiol.* 9: 867–874, 1977.
16. GREEN, J., D. T. YAMAGUCHI, C. R. KLEEMAN, AND S. MUALLEM. Selective modification of the kinetic properties of Na^+-H^+ exchanger by cell shrinkage and swelling. *J. Biol. Chem.* 263: 5012–5015, 1988.
17. GRINSTEIN, S., C. A. CLARKE, AND A. ROTHSTEIN. Activation of Na^+-H^+ exchange in lymphocytes by osmotically induced volume changes and by cytoplasmic acidification. *J. Gen. Physiol.* 82: 619–638, 1983.
18. GRINSTEIN, S., AND A. ROTHSTEIN. Mechanisms of regulation of the Na^+-H^+ exchanger. *J. Membr. Biol.* 90: 1–12, 1986.
19. GRINSTEIN, S., D. ROTIN, AND M. J. MASON. Na^+-H^+ exchange and growth factor-induced cytosolic pH changes. Role in cellular proliferation. *Biochim. Biophys. Acta* 988: 73–97, 1989.
20. GUPTA, I., V. K. JAIN, AND R. K. MISHRA. Studies on the effects of 2-deoxy-D-glucose on glucose uptake and glycolysis in respiratory-deficient yeast cells. *Indian J. Exp. Biol.* 19: 231–237, 1981.
21. HALL, E. J., AND L. ROIZIN-TOWLE. Biological effects of heat. *Cancer Res.* 44, *Suppl.*: 4708s–4713s, 1984.
22. HELMLE-KOLB, C., M. H. MONTROSE, G. STANGE, AND H. MURER. Regulation of Na^+-H^+ exchange in opossum kidney cells by parathyroid hormone, cyclic AMP and phorbol esters. *Pfluegers Arch.* 415: 461–470, 1990.
23. HINKE, J. A. M., AND S. G. A. MCLAUGHLIN. Release of bound sodium in single muscle fibers. *Can. J. Physiol. Pharmacol.* 45: 655–677, 1967.
24. HOEFER, K. G., AND N. F. MIVECHI. Tumor cell sensitivity to hyperthermia as a function of extracellular pH and intracellular pH. *J. Natl. Cancer Inst.* 65: 621–625, 1980.
25. INCE, C., B. THIO, B. VAN DUIJN, J. T. VAN DISSEL, D. L. YPEY, AND P. C. J. LEIJH. Intracellular K^+, Na^+ and Cl^- concentrations and membrane potential in human monocytes. *Biochim. Biophys. Acta* 905: 195–204, 1987.
26. KAHN, A. M., G. M. DOLSON, S. C. BENNETT, AND E. J. WEINMAN. cAMP and PTH inhibit Na^+-H^+ exchange in brush border membrane vesicles (BBM) derived from a suspension of rabbit proximal tubules (Abstract). *Kidney Int.* 25: 289, 1984.
27. LIU, F. F., J. K. POON, R. P. HILL, AND I. TANNOCK. Effect of hyperthermia on activity of the Na^+-H^+ antiport in EMT$_6$ cells. *Proc. Annu. Meet. Radiat. Res. Soc. 38th 1990*, p. 48.
28. MANCUSO, D., AND L. GLASER. Characterization of the Na^+-H^+ exchanger in human epidermoid carcinoma A-431 vesicles. *J. Cell Physiol.* 123: 297–304, 1985.
29. MICHELL, P. Conduction of protons through the membranes of mitochondria and bacteria by uncouplers of oxidative phosphorylation (Abstract). *Biochem. J.* 81: 24p, 1961.
30. MONTROSE, M. H., AND H. MURER. Regulation of intracellular pH in LLC-PK_1 cells by Na^+-H^+ exchange. *J. Membr. Biol.* 93: 33–42, 1986.
31. NG, L. L., AND C. DUDLEY. Intracellular pH clamping of human leucocytes: a technique for determination of cellular buffering power and Na^+-H^+ antiport characteristics. *Clin. Sci.* 77: 417–423, 1989.
32. O'NEILL, S. C., M. VALDEOLMILLOS, G. L. SMITH, AND D. A. EISNER. The effects of metabolic inhibition on intracellular pH and Ca. *Mol. Cell. Biochem.* 89: 199–203, 1989.
33. OVERGAARD, J. Influence of extracellular pH or the viability and morphology of tumor cells exposed to hyperthermia. *J. Intl. Cancer Inst.* 56: 1243–1250, 1976.
34. PERONA, R., AND R. SERRANO. Increased pH and tumorigenicity of fibroblasts expressing a yeast proton pump. *Nature Lond.* 334: 438–440, 1988.
35. POUYSSEGUR, J., C. SARDET, A. FRANCHI, G. L'ALLEMAIN, AND S. A. PARIS. Specific mutation abolishing Na^+-H^+ antiport activity in hamster fibroblasts precludes growth at neutral and acidic pH. *Proc. Natl. Acad. Sci. USA* 81: 4833–4837, 1984.
36. ROOS, A., AND W. F. BORON. Intracellular pH. *Physiol. Rev.* 61: 296–433, 1981.
37. ROTHENBERG, P., L. GLASER, P. SCHLESINGER, AND D. CASSEL. Epidermal growth factor stimulates amiloride-sensitive $^{22}Na^+$ uptake in A-431 cells. *J. Biol. Chem.* 258: 4883–4889, 1983.
38. ROTHENBERG, P., L. GLASER, P. SCHLESINGER, AND D. CASSEL. Activation of Na^+-H^+ exchanger by epidermal growth factor elevates intracellular pH in A-431 cells. *J. Biol. Chem.* 258: 12644–12653, 1983.
39. SIMCHOWITZ, L., AND A. ROOS. Regulation of intracellular pH in human neutrophils. *J. Gen. Physiol.* 85: 443–470, 1985.
40. SOKAL, R. R., AND G. F. ROHLF. *Biometry: The Principles and Practice of Statistics in Biological Research*. San Francisco, CA: Freeman, 1969, p. 776–790.
41. STOSCHECK, C. M., AND G. CARPENTER. Biology of the A-431 cells: a useful organism for hormone research. *J. Cell Biol.* 23: 191–202, 1983.
42. STOSCHECK, C. M., AND G. CARPENTER. Characterization of the metabolic turnover of epidermal growth factor receptor protein in A-431 cells. *J. Cell. Physiol.* 120: 296–302, 1984.
43. SUBJECK, J. R., AND T.-T. SHYY. Stress protein systems of mammalian cells. *Am. J. Physiol.* 250 (*Cell Physiol.* 19): C1–C17, 1986.
44. THOMAS, J. A., R. N. BUCHSBAUM, A. ZIMNIAK, AND E. RACKER. Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. *Biochemistry* 18: 2210–2218, 1979.
45. WEINER, I. D., AND L. L. HAMM. Regulation of intracellular pH in the rabbit cortical collecting tubule. *J. Clin. Invest.* 85: 274–281, 1990.
46. WEITZEL, G., U. PILATUS, AND L. RENSING. Similar dose response of heat shock protein synthesis and intracellular pH change in yeast. *Exp. Cell Res.* 159: 252–256, 1985.
47. YAJIMA, M., AND M. UI. Hydrocortisone restoration of the pH-dependent metabolic responses to catecholamines. *Am. J. Physiol.* 228: 1053–1059, 1975.
48. YI, P. N., C. S. CHANG, M. TALLEN, W. BAYER, AND S. BALL. Hyperthermia-induced intracellular ionic level changes in tumor cells. *Radiat. Res.* 93: 534–544, 1983.
49. ZHUANG, Y. X., E. J. CRAGOE, JR., T. SHAIKEWITZ, L. GLASER, AND D. CASSEL. Characterization of potent Na^+-H^+ exchange inhibitors from the amiloride series in A-431 cells. *Biochemistry* 23: 4481–4488, 1984.
50. ZUBAY, G. *Biochemistry* (2nd ed.). New York: Macmillan, 1988, p. 1162.